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# Effects of polyamines on the functionality of photosynthetic membrane in vivo and in vitro

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**Abstract**

The three major polyamines are normally found in chloroplasts of higher plants and are implicated in plant growth and stress response. We have recently shown that putrescine can increase light energy utilization through stimulation of photophosphorylation [Ioannidis et al., (2006) BBA-Bioenergetics, 1757, 821-828]. We are now to compare the role of the three major polyamines in terms of chloroplast bioenergetics. There is a different mode of action between the diamine putrescine and the higher polyamines (spermidine and spermine). Putrescine is an efficient stimulator of ATP synthesis, better than spermidine and spermine in terms of maximal % stimulation. On the other hand, spermidine and spermine are efficient stimulators of non-photochemical quenching. Spermidine and spermine at high concentrations are efficient uncouplers of photophosphorylation. In addition, the higher the polycationic character of the amine being used, the higher was the effectiveness in PSII efficiency restoration, as well as stacking of low salt thylakoids. Spermine with 50 μM increase $F_V$ as efficiently as 100 μM of spermidine or 1000 μM of putrescine or 1000 μM of $Mg^{2+}$. It is also demonstrated that the increase in $F_V$ derives mainly from the contribution of PSIIα centers. These results underline the importance of chloroplastic polyamines in the functionality of the photosynthetic membrane.


*Keywords:* Non-photochemical quenching; Chloroplast; Cations; Stress; Proton motive force; ATP

## 1. Introduction

Polyamines (PAs) are low molecular weight biogenic amines, ubiquitous in all organisms. The three main PAs differ in the number of positive charges exhibited in the physiological pH of the cell [two in putrescine (Put), three in spermidine (Spd) and four in spermine (Spm)]. Thus PAs can interact with anionic macromolecules like DNA, RNA, acid phospholipids and proteins [1–3]. In plants, they have been implicated in a wide range of growth and developmental processes such as cell division, dormancy breaking of tubers and germination of seeds, stimulation, support and development of flower buds, embryogenesis, fruit set and growth, fruit ripening, plant morphogenesis and response to environmental stresses [4]. A striking increase of polyamines occurs under deprivation of cations such as $K^+$ and $Mg^{2+}$ (for a review see ref. [5]). Although, according to evidence, polyamines seem to be important growth regulators, their precise physiological function and mechanism of action still remain unclear.

Substantial evidence has been accumulated concerning the role of PAs in the chloroplasts. Dörnemann et al. [6] has demonstrated that PAs are down regulated during the biogenesis of the photosynthetic apparatus. PA biosynthesis is controlled by light [7] and during photoadaptation the Spm/Put ratio is correlated to the structure and function of the photosynthetic apparatus [8,9]. The primary photoreceptor for long term changes, such as photoadaptation, has been reported to be the PSII [9]. Manipulation of endocellular polyamine level can increase plant (photosynthetic) tolerance during ozon stress [10], UVB stress

*Abbreviations:* Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Put, Putrescine; PAR, photosynthetically active radiation; PAs, polyamines; NPQ, non-photochemical quenching; $F_V/F_M$, maximum photosynthetic efficiency; $F_V=F_M-F_0$, maximum variable fluorescence; $F_M$, maximum fluorescence (dark); $F_M'$, maximum fluorescence (light); $F_0$, initial fluorescence; LEF, linear electron flow; Fecy, Ferricyanide; MV, methylviologen; $\Delta\psi$, electrical component of the proton motive force; ATP, adenosine triphosphate; HEPES, (N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid); Tricine, (N-tris-(hydroxymethyl)methylglycine)

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[11], chilling [12] and salt stress [13]. Also it is reported that Spm and Spd in vitro lead to disassembly of the oxygen evolving complex, while Fourier transformed infrared difference spectroscopy has showed major alterations of the proteins of photosystem II (PSII) secondary structure as positive charge increases [14–16].

We have recently revealed the regulatory role of the free Put pool in photophosphorylation [17]. The regulation of ATP production is one of the most important processes for the photosynthetic organism which determines the amount of energy available for energy-consuming processes. On the other hand, regulation of ATP synthesis via regulation of putrescine levels, increases our understanding on the puzzling role of this diamine, thus leading to the following question: what are the stimulating properties of the other major polyamines? With regard to the present work, the link between energy regulation and polyamine level is further investigated by comparing the effects of the three PAs. On the grounds that polyamines could act as cations, this work also includes the determination of rather overlooked coulombic effects of PAs on the photosynthetic membrane. The whole study follows a top-down approach, which begins with the monitoring – by means of chlorophyll fluorescence – of the status of photosynthetic membrane of leaf discs treated with PAs. Interestingly, it is the first time the stimulation of non-photochemical quenching (NPQ) by biogenic amines has been reported.

## 2. Materials and methods

### 2.1. Plant material

Tobacco plants (*Nicotiana tabacum* cv. Xanthi) grown in a greenhouse for 6–8 weeks were transferred for 2 days in a thermostated chamber (25 °C) under continuous light (50 μmol photons $m^{-2}$ $s^{-1}$). For the purpose of leaf discs experiments, healthy leaves were cut with a special knife. Discs of 10 mm diameter freely floated in petri dishes at 25 °C with or without PAs treatment and illuminated for 16 h with 50 μmol photons $m^{-2}$ $s^{-1}$. PAs stock solutions (Sigma) were freshly made and the working concentration was 1 mM. Tobacco was substituted with market swiss-chard (*Beta vulgaris*) in photophosphorylation experiments.

### 2.2. Chloroplast isolation

Chloroplasts were isolated by grinding leaves of tobacco (*Nicotiana tabacum* cv. Xanthi) in 50 mM Tricine buffer, pH 7.6 (adjusted with KOH), containing 0.33 M sucrose. The slurry was filtered through cheesecloth and chloroplasts were precipitated by centrifugation at 3000×*g* for 7 min. The pellet was resuspended in a small aliquot of the isolation medium (chlorophyll up to 2 mg $ml^{-1}$). The reaction mixture was a low cation, low osmoticum buffer (100 mM sorbitol, 10 mM Tricine pH 7.6 adjusted with KOH) suitable for evaluation of the cationic effect of PAs on thylakoid membranes. Chlorophyll concentration was estimated according to Holden [18].

### 2.3. Fluorescence measurements

In order to determine the maximum photosynthetic efficiency ($F_V/F_M$) in vivo, samples were dark adapted for 15 min and fluorescence was measured using a Handy-PEA Fluorophotometer (Hansatech, King's Lynn, UK). For determination of non-photochemical quenching ($NPQ=F_M/F'_M-1$) [19] leaf discs were illuminated with red light of different intensities (2, 30, 60, 100, 200, 400, 500, 800, 1200, 1800 μmol photons $m^{-2}$ $s^{-1}$) for 5 min using Handy-PEA (multi-hit mode) and a saturating pulse of 3000 μmol photons $m^{-2}$ $s^{-1}$ was applied for determination of $F'_M$. $NPQ_{fast}$ [$NPQ_{fast}=(F_M/F'_M-1)-(F_M/F'_{MR})$] was determined according to Maxwell and Johnson [20]. For $F'_{MR}$ the value after 2 min of relaxation in the dark from a single saturating pulse was taken. For calculation of fluorescence relaxation in the dark (Fig. 2E) was used the parameter $(F_M-F_{Mtd})/F_M$ [21]. $F_M$ is the maximal fluorescence amplitude observed upon application of a light saturation pulse after complete relaxation and $F_{Mtd}$ is the value at time *t* after darkening. After the 5-min induction period of NPQ continuous light (500 μmol photons $m^{-2}$ $s^{-1}$) was switched off and only saturating pulses were applied every 20 s for 5 more min.

Fluorescence spectroscopy was performed in a similar way for in vitro experiments. More specifically, samples were dark adapted for 2 min and fluorescence was measured by means of using the Handy-PEA Fluorophotometer (Hansatech, King's Lynn, UK). Additives such as PAs and inorganic salts were incubated for 2 min in the dark. DCMU was added for electron transport inhibition after $Q_A$ and light intensity used was 1500 μmol photons $m^{-2}$ $s^{-1}$. PSIIα and PSIIβ centers were calculated from the slope of $\ln[(A_{mx}-A_t)/A_{mx}]$ according to the method of Melis [22]. $A_{mx}$ is the complementary area in the DCMU curves and $A_t$ the Area at time *t*.

### 2.4. Polarographical measurements

Polarographical measurements were carried out using a Clark type electrode, as previously described [11]. Coupled linear electron flow (LEF) and PSII activity of tobacco chloroplasts supported by methylviologen (MV) and ferricyanide (Fecy) [23], respectively, were measured under 1000 μmol photons $m^{-2}$ $s^{-1}$ in a reaction medium containing 100 mM sorbitol, 10 mM Tricine, 2 mM ADP, 2 mM $Na_2HPO_4$, (pH 7.6 adjusted with KOH). Experiments were carried out three times in different tobacco preparations with similar results.

### 2.5. Photophosphorylation measurements

Chloroplasts were extracted from healthy leaves of swiss-chard as in ref. [24]. Chloroplasts were prepared in a grinding buffer containing 0.4 M sucrose, 40 mM tricine, 10 mM NaCl, 10 mM sodium ascorbate, (pH 7.8 adjusted with KOH). After grinding, the suspension was filtered and chloroplasts were pelleted by centrifugation. Preparations obtained by this treatment were stable – in terms of photophosphorylation – for at least 8 h. Photophosphorylation was measured in a medium containing 200 mM sorbitol, 12 mM HEPES, 10 mM tricine, 10 mM KCl, 5 mM $MgCl_2$, 2 mM $K_2HPO_4$, 1 mM sodium ascorbate and 0.6 mM ADP (pH 7.8 adjusted with KOH). 300 nM valinomycin (in ethanol, final concentration 0.25%) were added for the dissipation of $K^+$ gradients, while phenazine methosulphate (40 μM) was the electron acceptor. PAs stock solution was freshly prepared and the incubation time in the dark was 1 min. Illumination was provided for 3 min in water-jacked chamber (thermostated at 23 °C) under continuous stirring and the reactions were stopped after switching off the light by mixing with 10% $HClO_4$ (final pH<1). ATP content of the supernatant was determined, after neutralization with KOH and centrifugation in order to pellet the remnants of the thylakoids and $KClO_4$, by means of a sensitive kit using the luciferin-luciferase system (FL-ASC Kit, Sigma) in a LS50-B Luminometer Spectrometer (Perkin Elmer) according to the manufacturer's instructions. The quantification was carried out by adding 3 times small amounts of ATP stock solution of known concentration (freshly prepared). The preparations were essentially free of adenylate kinase activity as previously described [24]. The results were validated for three different swiss-chard preparations.

## 3. Results

### 3.1. NPQ induction and relaxation in different light intensities for polyamine treated tobacco leaves

The role of PAs in photoadaptation is well established [9,25]. However, the role of PAs in short-term phenomena such as the photoprotection mechanisms relative to chlorophyll a fluores-

cence quenching, are not so well studied. For the in vivo study of the role of PAs in the general framework of light energy utilization by the photosynthetic apparatus, exogenously supplied PAs manipulated the intracellular levels. Exogenous addition of 1 mM polyamines in tobacco leaf discs and 16 h incubation were selected respectively for the working concentration and the incubation period after preliminary experiments (i.e. 0.1 up to 3 mM for PAs and 8 h up to 72 h for incubation period were tested; data not shown). Leaf discs were illuminated for 5 min with different light intensities covering the major ambient light categories (i.e. low: LL, medium: ML and high light: HL) and their maximal fluorescence were quantified and used for NPQ calculation [19]. Normally, the photosynthetic apparatus increases thermal dissipation with increasing light intensity (Fig. 1A). Spd increases NPQ in low light intensities up to 260% (Fig. 1C) and Spm increases NPQ up to 170% (Fig. 1D) in comparison to the untreated control. On the contrary, Put decreases NPQ in low light intensities, an effect that is reversed in ML (Fig. 1B). All three PAs decrease NPQ in higher light intensities (Figs. 1B–D).

The differences observed in NPQ between the control and polyamine-treated leaf discs encouraged us to further study this stimulatory phenomenon. $NPQ_{fast}$ is a measure of the quenching due to membrane energization and state transition [20]. In untreated leaf discs, $NPQ_{fast}$ increases with proportion to light intensity in LL and ML but in HL (>1000 μmol quanta·$m^{-2}$·$s^{-1}$) reaches a plateau (Fig. 2A). Spd and Spm increase $NPQ_{fast}$ about 2.5 times (Fig. 2C) and about 4 times (Fig. 2D) respectively. This increase is evident in LL. In Put treated leaf discs $NPQ_{fast}$ is decreased in LL and HL whereas this effect is reversed in ML (Fig. 2B). In all cases treatment decreases $NPQ_{fast}$ in HL (Figs. 2B–D). This could be partly due to the photoinhibition caused by high light.

More information concerning thylakoid membrane relaxation is provided through the $(F_M - F_{Mtd})/F_M$ [21]. In untreated leaf discs (control) energized for 300 s with a light intensity of 500 μmol quanta·$m^{-2}$·$s^{-1}$ the parameter in question decreases rapidly for the first 40 s mainly due to movements of protons from lumen to stroma (Fig. 2E). In the case of Put about 68% of total NPQ has relaxed within 40 s. The rest 32% relaxes at about 240 s of dark incubation, whereas the control relaxes at about 120 s. Spd and Spm treatment under these experimental conditions do not seem to alter relaxation kinetics (Fig. 2E).

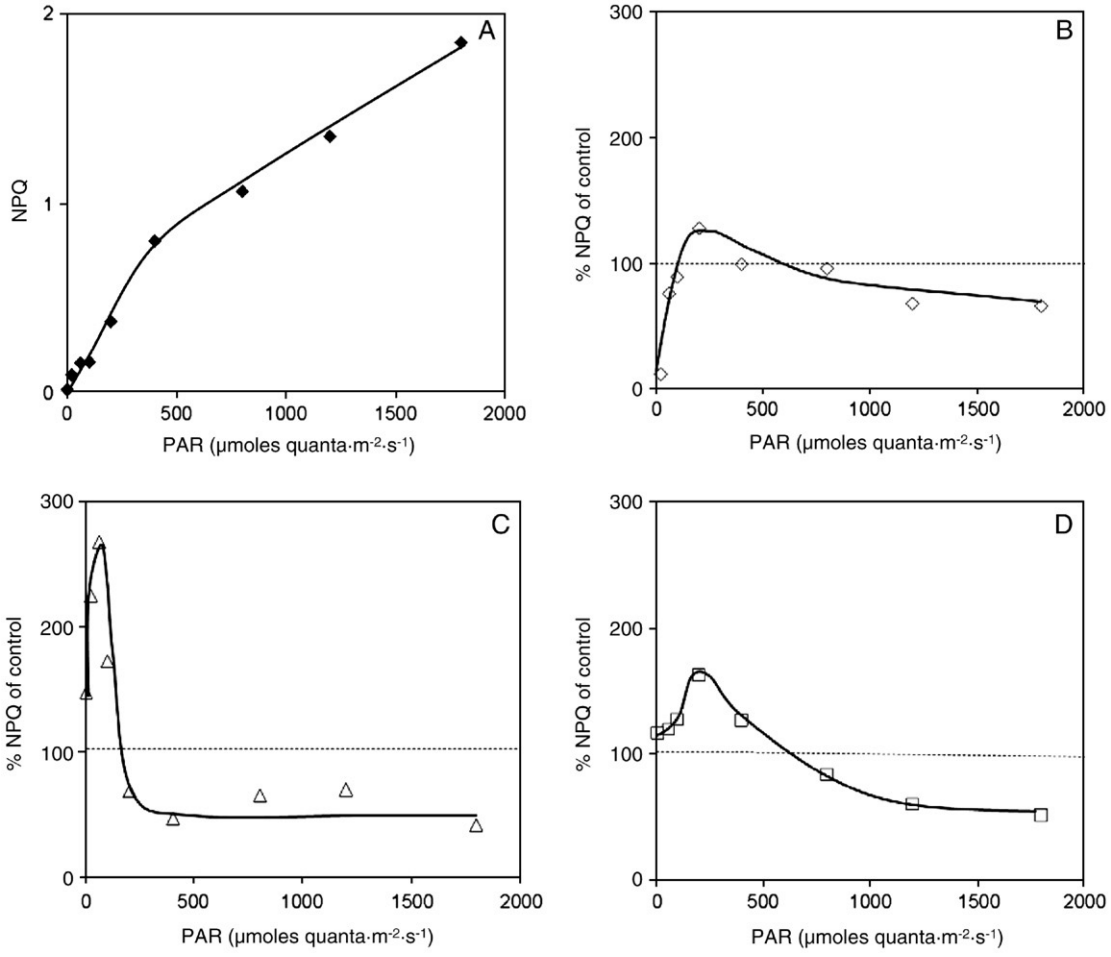


Fig. 1. Light intensity dependency of NPQ in tobacco leaf discs treated and not treated with PAs. (A) Control absolute values for NPQ after 5 min illumination in different light intensities. Control values were arbitrarily set as 100 (dashed line) and the values of treated leaf discs are illustrated as % difference. Leaf discs were treated with 1 mM Put (B), 1 mM Spd (C) and 1 mM Spm (D). Leaf discs were incubated with PAs for 16 h in 50 μmol $m^{-2}$ $s^{-1}$.

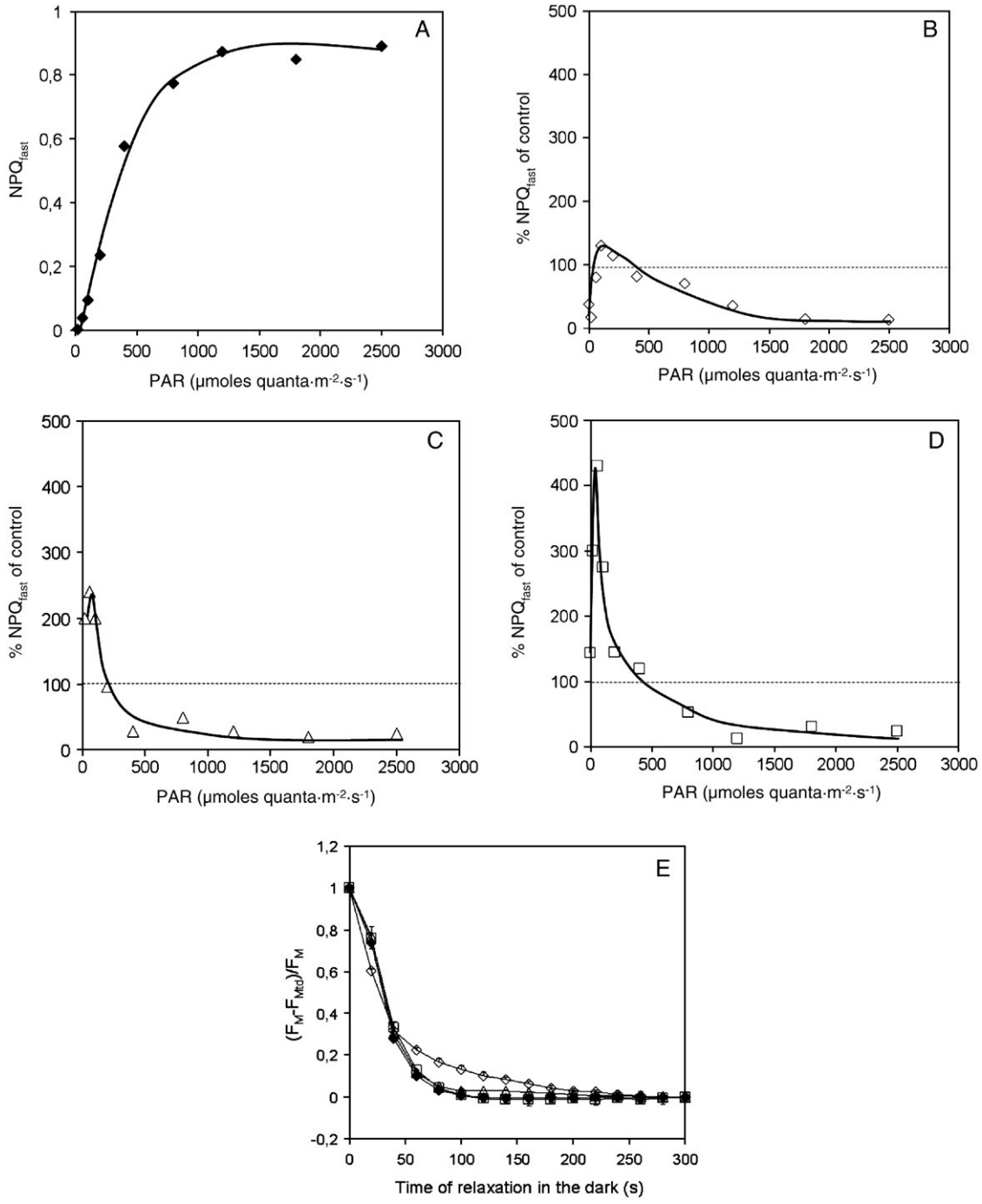


Fig. 2. Light intensity dependency of $NPQ_{fast}$ in tobacco leaf discs treated and not treated with PAs. (A) Control absolute values for $NPQ_{fast}$ after 5 min illumination in different light intensities. Control values were arbitrarily set as 100 (dashed line) and the values of treated leaf discs are illustrated as % difference. Leaf discs were treated with 1 mM Put (B), 1 mM Spd (C) and 1 mM Spm (D). Leaf discs were incubated with PAs for 16 h in 50 μmol $m^{-2}$ $s^{-1}$. (E) Relaxation kinetics for membrane energization. The parameter $(F_M - F_{Mtd})/F_M$ estimates the relaxation of the thylakoid membrane mainly due to the reduction of ΔpH between lumen and stroma. Light (500 μmol $m^{-2}$ $s^{-1}$ was switched off at 0 s and values for $F_{Mtd}$ were recorded every 20 s (the intensity of the saturation pulse used was 3000 μmol $m^{-2}$ $s^{-1}$ of 0.4 s duration). Put (open diamonds) delays relaxation after 40 s whereas Spd (open triangles) and Spm (open squares) have marginal effects in comparison to the control (closed diamonds) under these experimental conditions. The values of panel E were normalized to 1 for easier comparison. The absolute values of $(F_M - F_{Mtd})/F_M$ (at $t=0$ s) for control, Put, Spd and Spm were 0.471±0.022, 0.210±0.015, 0.424±0.018 and 0.443±0.019 respectively ($n=3$).

### *3.2. Coulombic effect of polyamines on photosynthetic membrane*

Previous section made it clear that manipulation of polyamine levels affects NPQ. In an attempt to increase our understanding on the role of PAs in such phenomena, a series of *in vitro* experiments were performed with the use of isolated chloroplasts. Typical isolation procedures deplete small solutes like polyamines. Therefore this natural amine has to be added exogenously for the simulation of the *in vivo* conditions. Low

salt media were used for isolation and resuspension (see Materials and methods). Consequently, the experiment could also assess the cationic effect of PAs that would otherwise be masked by high cationic buffers. Low photosynthetic efficiency exhibited by such preparations is easily restored, by addition of monovalent or divalent cations, which cause stacking of thylakoids [26]. Different amounts of PAs, monovalent and divalent inorganic cations were tested for their ability to increase $F_V/F_M$; the results are illustrated in Fig. 3. Spm and Spd are the most efficient ones in $F_V/F_M$ restoration reaching 60% and 70% respectively. The inorganic cations and Put increase the $F_V/F_M$ ratio approximately 40%. Interestingly, higher amounts of Spm and/or Spd reverse the effect and lead to a decline of $F_V/F_M$. This is in line with a recent work showing that Spd and Spm have an inhibitory effect in oxygen evolving complex [15]. This inhibition could not be overcome by $CaCl_2$ as it is usually the case with toxic divalent cations such as mercury [15,27,28].

DCMU inhibits electron transport and the same measurement (fluorescence induction) quantifies a) the rate that the reaction centers of PSII close after a single reduction of $Q_A$ in the presence of DCMU and b) the rate reaction centers close when the whole PQ pool is reduced (such as in Fig. 3) in the absence of DCMU. DCMU poisoned thylakoids incubated in the dark result in a characteristic sigmoidal fluorescence signal upon illumination (0 μM curves in Figs. 4A–D). The addition of selected amounts of PAs or of $Mg^{2+}$ leads to a significant increase of maximal fluorescence. In Fig. 4 the detailed kinetics for the four different treatments are illustrated: treatment with additional Put (Fig. 4A), Spd (Fig. 4B), Spm (Fig. 4C) and $Mg^{2+}$ (Fig. 4D). For each cation four different concentrations were tested (50 μM, 100 μM, 500 μM and 1000 μM). The inorganic cation was selected both as an external control, as well as a measure of effectiveness, also allowing comparison with previous works (Fig. 4D). For instance, a value 2.5 for $F_M$ (values are normalized to $F_0$) is feasible with either 1000 μM of Put, or 100 μM of Spd, or 50 μM of Spm or 1000 μM of $Mg^{2+}$ (Fig. 4). Due to the difference of the same concentration of the polyamines in effectiveness regarding fluorescence increase, we evaluated maximal restoration based on the results of Fig. 4. In Fig. 5 it is demonstrated that $F_V$ in DCMU poisoned samples is increased 60% by 0.3 mM of Spm, 70% by 4 mM of Spd, 35% by 10 mM of Put and about 50% by a combination of inorganic cations (10 mM $MgCl_2$ and 30 mM KCl).

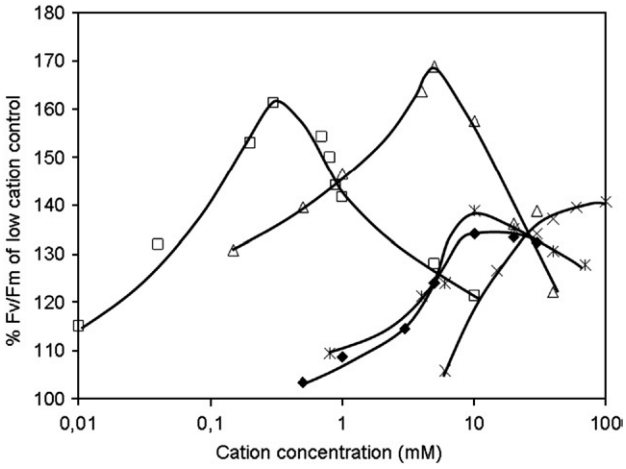


Fig. 3. Restoration of $F_V/F_M$ in low salt thylakoids. Dose course for Put (diamond), Spd (triangle), Spm (square), $K^+$ (cross) and $Mg^{2+}$ (asterisk). Thylakoids were incubated for 2 min with the corresponding cation. The *x* axis is logarithmic for clarity and $F_V/F_M$ value for control was 0.393±0.016 ($n$=3). The tetramine Spm is far more effective than the other salts. The pH of the medium was 7.6 and the concentration of Chl was 10 μg $ml^{-1}$.

### 3.3. The effect of polyamines on PSIIα and PSIIβ

Polyamines increase the amplitude of maximal fluorescence of low salt thylakoids, and Spm, in particular, is much more effective than inorganic cations. The heterogenicity of PSII populations regarding their antenna size led us to check if polyamines evenly affect the different subpopulations. There are PSII with a large antenna (PSIIα) and PSII with a smaller one (PSIIβ) [21]. Polyamines (used in concentrations similar to those of Fig. 5) improve PSIIα constant five times (Fig. 6B) whereas they have a minor impact in PSIIβ centers (Figs. 6A, C). Higher efficiency resulted in higher oxygen evolution rates for PSII. Put increased up to 3 times Fecy supported oxygen evolution (Figs. 7A, B), whereas Spd and Spm showed even better stimulations reaching 3.5 and 4 times respectively (Figs. 7C, D). The increase in oxygen evolution is obvious from the first millimolar of added polyamine. Put dose curve does not exhibit the steep increase observed in the case of Spd. This is also true for the corresponding curves in the case of the linear electron flow (Fig. 8). LEF is increased 2-fold by Put (Fig. 8A), 2.4-fold by Spd (Fig. 8C) and 2.1-fold by Spm (Fig. 8D). $Mg^{2+}$ was used again as reference cation and exhibited a 2-fold increase (Fig. 8B).

### 3.4. Effect of polyamines on photophosphorylation

Finally, the steady state phosphorylation was evaluated in the presence of polyamines. In these experiments media of optimal cationic content were used, so that the results highlight specific actions of polyamines, not necessarily relative to the results of Figs. 3–8. Moreover, on condition that photophosphorylation is easily biased in vitro by adenylate kinase activity, it would be ideal that preparations have a negligible adenylate kinase activity. This was not possible with the tobacco chloroplasts, so we searched for a better experimental system trying to avoid the use of inhibitors like di(adenosine-5)pentaphosphate. *Beta vulgaris* chloroplasts did not exhibit adenylate kinase activity in agreement with previous reports [23]. Another source of errors in an artificial phosphorylation system is an ion gradient due to $K^+$ of the medium. For the dissipation of $K^+$ gradients that could influence phosphorylation, valinomycin was included in the phosphorylation medium. PAs increase steady state phosphorylation rate in a complex manner (Fig. 9). Put stimulates up to 70% ATP synthesis (Fig. 9A), Spd induces a marginal increase if any (Fig. 9B) and Spm stimulates about 30% photophosphorylation (Fig. 9C). Interestingly, both Spd and Spm at higher

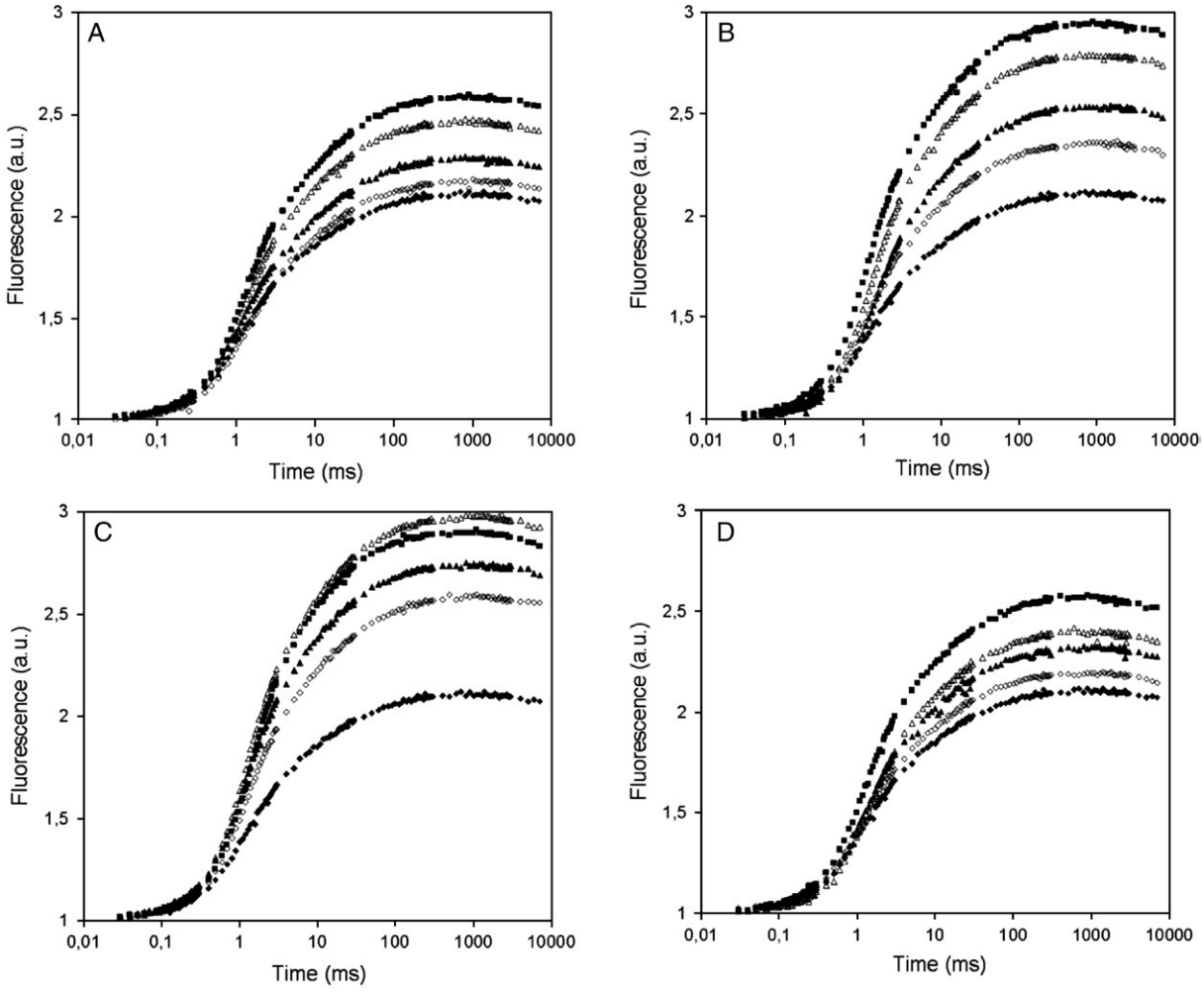


Fig. 4. Detailed kinetics of fluorescence rise F of low salt thylakoids in the presence of DCMU for different concentrations of Put (A), Spd (B), Spm (C) and $Mg^{2+}$ (D). The concentrations used were 0 μM (closed diamond), 50 μM (open diamond), 100 μM (closed triangle), 500 μM (open triangle) and 1000 μM (closed square). DCMU inhibits electron transport after $Q_A$ and therefore the information of the signal is independent of the PQ pool size in contrast to the $F_V/F_M$ values of Fig. 3 where the electron transport operated. The Chl concentration was 10 μg $ml^{-1}$ and all the substances were added as chloride salt. The addition of DCMU increased Fo about 15% and the time axis is logarithmic for clarity.

concentrations uncoupled electron transport from phosphorylation. On the contrary, Put even at high concentrations (15 mM) stimulated about 50% ATP synthesis.

## 4. Discussion

In this contribution we investigated the effect of polyamines on the photosynthetic membrane both in vivo and in vitro. Polyamines occur normally in the chloroplast of higher plants and algae and their role in long term phenomena, such as photoadaptation, is well documented [9,25]. Here we report that polyamine supply caused important effects on light energy dissipation mechanisms. Spm and Spd increased the NPQ many times, whereas Put was less effective (Fig. 1). NPQ is the sum of three processes (a) membrane energization, (b) state transitions and (c) photoinhibition [29–32]. In an attempt to focus on the first two processes we measured $NPQ_{fast}$ (Fig. 2). Higher NPQ values for polyamine treated leaf discs compared to the control values during the induction period are followed by high $NPQ_{fast}$ values after switching off continuous illumination (Fig. 2). $NPQ_{fast}$ is mainly due to proton efflux from lumen to stroma and due to the higher $t_{1/2}$ relaxation time for state transitions only a small contribution of qT is anticipated [20,21]. The latter implies that the differences being observed in NPQ and in $NPQ_{fast}$ are mainly due to qE. This is in agreement with previous results in isolated chloroplasts where tertiary amines increased qE at low light intensities [33]. In both cases (tertiary amines and polyamines) the effect was inversed at higher light intensities. To the best of our knowledge there is no published mechanism that could interpret this stimulation of NPQ or qE by amines, although localized proton potential was earlier proposed to be involved [34].

On the other hand, there are indications that polyamines affect proton pools near or/and in the photosynthetic membrane (Fig. 9). Polyamines, due to their proton sequestering capacity, might accept protons from ‘producers’ (i.e. PSII, $Cyt_{b6f}$, PSI) and deliver protons to ‘consumers’ (ATPase). This could explain why polyamines stimulate ATP synthesis during light pe-

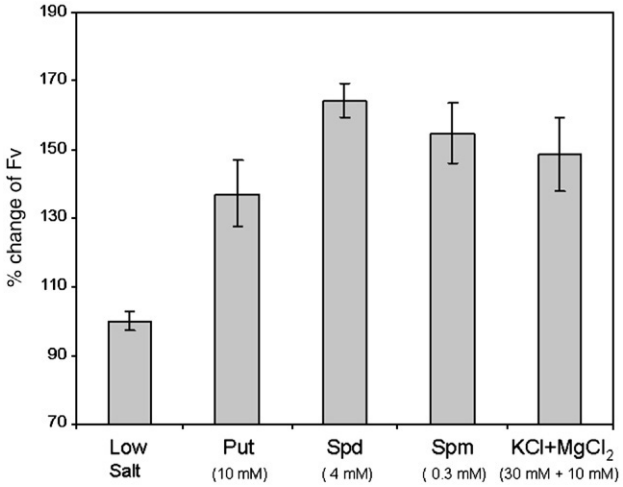


Fig. 5. Comparison of the maximal restoration of low salt thylakoids in terms of $F_V$ for each PA in the presence of DCMU. The addition of DCMU increased Fo about 15%. After dose-course experiments the values that secured maximal restoration for PA and inorganic cations (combination of monovalent and divalent cations) are illustrated. Conditions as in Fig. 4. Standard deviation is represented by vertical bars ($n=4$).

riods (Fig. 9). Stimulation of ATP synthesis by low molecular weight amines is a well documented phenomenon [35–37]. It has only recently been demonstrated that this kind of stimulation is feasible by the biogenic Put [17]. Present study offers a comparison among different stimulation properties of PAs concerning ATP synthesis. The relative efficiency in decreasing order is Put > Spm ≥ Spd (Fig. 9).

The mechanism of stimulation might not be necessarily the same for Put and Spm. At least two differences are obvious. In Spm, (a) the concentration needed for the stimulation is much lower and (b) Spm can also uncouple electron transport. Horner and Moudrianakis [38] who studied photophosphorylation in the presence of permeable amine buffers reported delays in lumen discharging. Under our experimental conditions delays are obvious only in Put treatment after the first 40 s of relaxation and are related to a minor fraction (about 32%) of total NPQ. In the case of Spm a different kind of stimulation mechanism may be valid, as it will be discussed later on.

Put stimulates chemiosmotic ATP synthesis even at concentration of 15 mM, twice as high as those previously tested. Yet maximal stimulation is observed at concentrations near 1 mM in agreement with our previous report [17]. On the contrary, Spd and Spm proved to be efficient uncouplers of photophosphorylation at higher concentrations. This could compensate for the loss of PSII efficiency (Fig. 3) at higher concentration and explain the increase in oxygen evolution (Fig. 7). Uncoupling can be of biological importance because it relieves membrane energization and regulates ROS formation [39]. Both the stimulation of ATP synthesis at low concentrations of amines and the uncoupling at higher doses were also demonstrated for ammonia, methylamine, ethylamine, imidazole and hexylamine [35–37]. Remarkably, a few μM of hexylamine in ref [37] or Spm (this work) are capable of stimulating ATP synthesis. The low concentration (μM) of Spm needed for stimulation, might be sufficient to bridge proton circuits inside the thylakoid membrane and deliver protons to the ATPase as it was previously suggested for hexylamine under similar experimental conditions [37].

Moreover, polyamines exhibit a sound cationic behavior at pH values of 7.6 (Figs. 3–8). This coulombic effect of Put, Spd and Spm was anticipated due to their proton sequestering groups (amino and imino groups) and their high pKs. However,

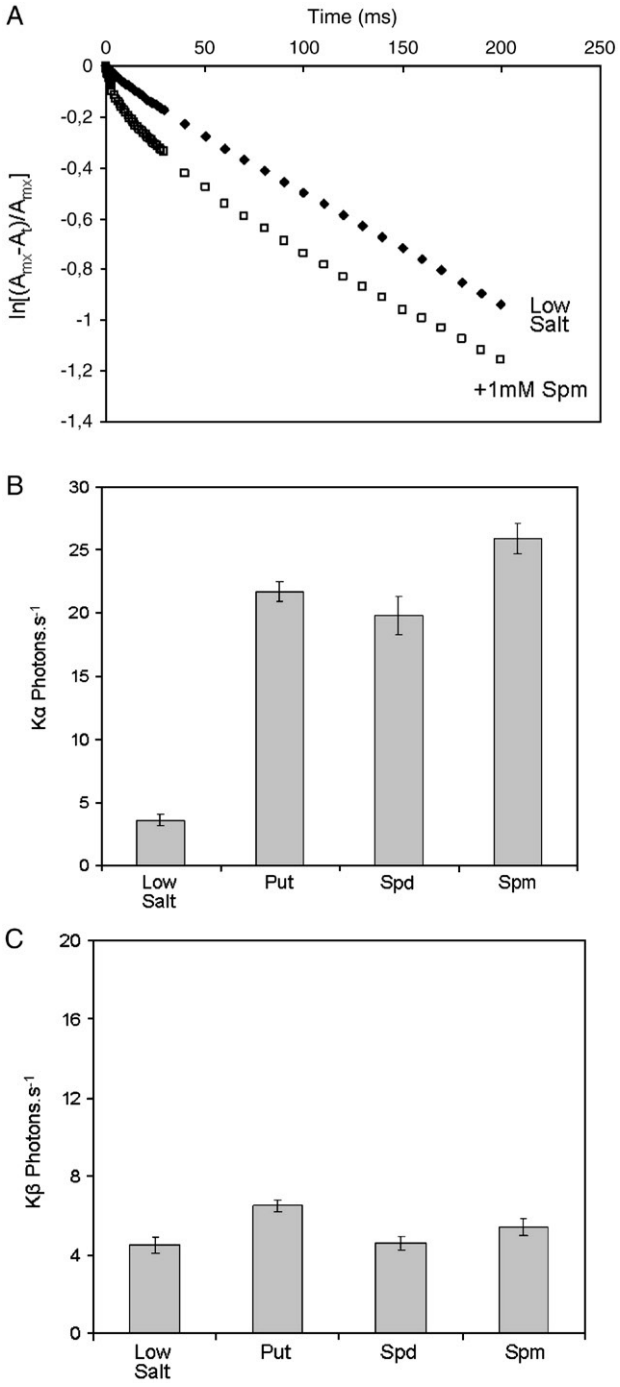


Fig. 6. The influence of PAs on the restoration of PSII activity for the different populations (α and β) of PSII. (A) The detailed kinetics of the $\ln[(A_{max}-A)/A_{max}]$ for low salt and thylakoids treated with 1 mM Spm. The influence of 10 mM Put, 4 mM Spd and 0.3 mM Spm in Kα (B) and Kβ (C). Experimental conditions as in Fig 4.

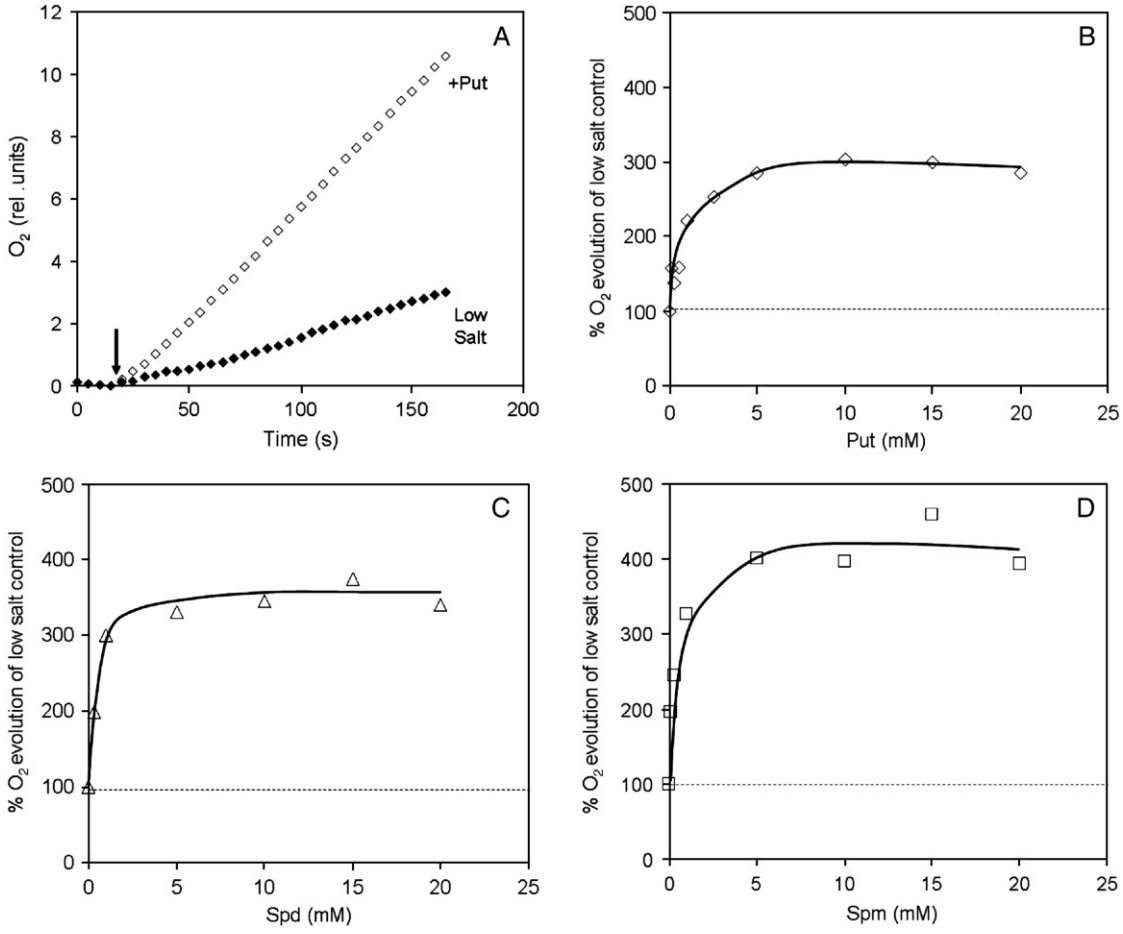


Fig. 7. (A) Polarography of PSII activity in the presence and absence of 10 mM Put. Illumination started at 20 s (arrow), the light intensity was 1000 μmol $m^{-2}$ $s^{-1}$. Stimulation of PSII activity by different concentrations of Put (B), Spd (C) and Spm (D) in low-salt tobacco chloroplasts. Polarographical measurements performed in a reaction medium containing 100 mM sorbitol, 10 mM Tricine, 2 mM ADP, 2 mM $Na_2HPO_4$, pH 7.6/KOH. Control values (100%) in low salt (0 mM salt added) under 1000 μmol $m^{-2}$ $s^{-1}$ were 82 μmol $O_2$ $[mgChl]^{-1}$ $h^{-1}$ and the electron acceptor was 3 mM Fecy. Standard deviation was less than point size.

their cationic effect was neither quantified nor compared to the efficiency of inorganic cations such as $K^+$ or $Mg^{2+}$ that normally occur in chloroplasts and are tightly correlated with many chloroplastic processes. This overestimates the in vivo role of inorganic cations in photosynthesis because it underestimates or neglects coulombic effects deriving from other sources. On the grounds that major fraction of chloroplastic cations are nitrogen containing organic compounds, such as polyamines, we should keep in mind what their effective range of concentration is.

Spm and Spd exhibit significant restoration of maximum photosynthetic efficiency and the relative efficiency in decreasing order is [Spm] > [Spd] > [Put] ≥ [$Mg^{2+}$] > [$K^+$] (Fig. 3). Such results are in line with previous reports regarding the effect of $K^+$ and $Mg^{2+}$ (for a review, see Barber [40]). The stimulation in question could be explained through a spatial segregation of the slow PSII and the fast PSI (it has approximately three times as fast trapping time as PS II) driven by electrostatic screening that is correlated to stacking of thylakoid membranes and a decrease in spillover [40,41]. The data from low salt thylakoids are consistent with a recent work reporting that PSII has the capability for self-organization when transferred from low to high salt conditions [42]. The latter also suggested that a disconnection of LHCII trimers from PSII occurs under low salt conditions, which is in line with our results and will be discussed in conjunction with cationic effects of polyamines.

Furthermore, for first time it is demonstrated that polyamines are 1–2 orders of magnitude more efficient in restoring photosynthetic efficiency than inorganic cations. By inhibition of the electron transport at the step of $Q_B$ we studied the effect of polyamines and inorganic cations on PSII (Fig. 4). Interestingly, 50 μM Spm have the same effect as 20 times more $Mg^{2+}$ does. Under our experimental conditions Spm has a total charge near +4; as a result, one might expect a similar effect with twice as much $Mg^{2+}$ (i.e. 100 μM). The latter was not true implying that it is not only the total charge that matters, but also other factors, such as the specificity in the binding between the cation and the substrate. This is also the case for Spd, which at 4 mM has a comparable effect with the combination of 30 mM $K^+$ plus 10 mM $Mg^{2+}$ (Fig. 5). Not surprisingly, Put action resembles more that of a divalent inorganic cation (Figs. 3–8). Furthermore, cationic effects are known to derive from stacking of thylakoid membranes [26]. PSIIα centers occur in grana, whereas PSIIβ ones are found in unstacked regions [22]. In line with this view, polyamines affected mainly PSIIα centers indicating that antenna of PSII

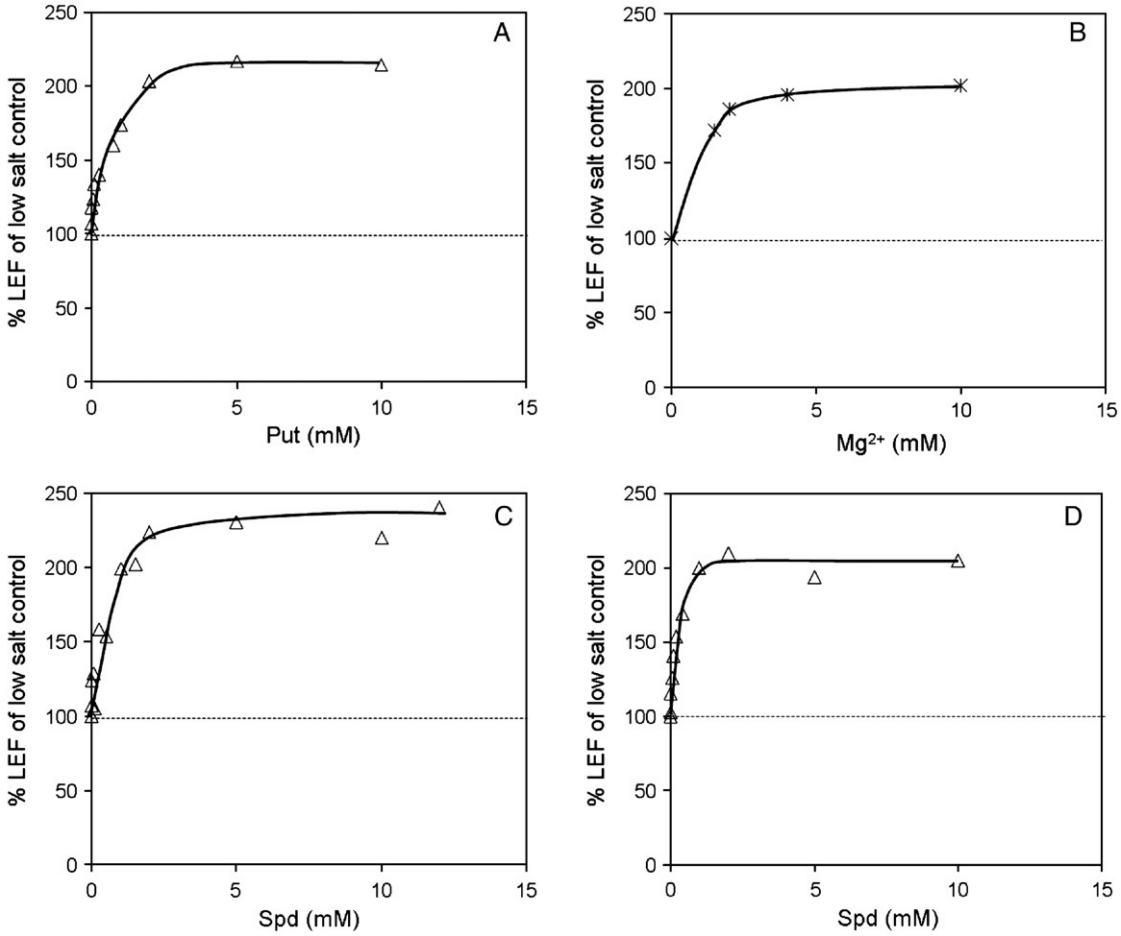


Fig. 8. Stimulation of linear electron flow (LEF) by different concentrations of Put (A), inorganic cations (B), Spd (C) and Spm (D) in low-salt tobacco chloroplasts. Polarographical measurements performed in a reaction medium similar to that of Fig. 7. Control values (100%) in low salt (0 mM salt added) under 1000 μmol $m^{-2}$ $s^{-1}$ were 76 μmol $O_2$ $[mgChl]^{-1}$ $h^{-1}$ and the electron acceptor was 100 μM MV. Standard deviation was less than point size.

is prerequisite for the establishment of the interaction. It is well documented that the LHCII protein is abundant in thylakoids and its surface is negatively charged [43]. Hence, cations like polyamines can mask charges and allow stacking of neighboring membranes. Kirchhoff et al. [42] have recently shown that incubation of thylakoids under unstacking conditions leads to intermixing and randomization of the protein complexes, accompanied by disconnection of LHCII trimers from PSII and a decreased connectivity between PSIIα centers. In good agreement with our results this effect is reversed upon cation addition [42]. The stacking effect and the resegregation of the protein complexes benefits both PSII oxygen evolution ability and linear electron flow in all cases tested (Figs. 7, 8). Once again, the relative efficiency was [Spm] > [Spd] > [Put] ≈ [Divalent inorganic cations]. The stimulation of PSII activity by polyamines and inorganic cations is in line with many previous reports from 1970s and 1980s (e.g. see ref [44,45]). Picosecond fluorescence kinetics in spinach chloroplast showed that the addition of $Mg^{2+}$ to broken spinach chloroplasts isolated in the absence of $Mg^{2+}$ has two effects which occur at different concentration ranges [46]. As the $Mg^{2+}$ concentration is increased from 0.0 to 0.75 mM, the rate constant for transfer between PS II and PSI decreases [46]. As the $Mg^{2+}$ concentration is further increased up to 2.0 mM, there are changes in the Chl *a/b* light-harvesting antenna, which both increase the absorption cross-section of PS II and bring about communication between PS II units [46]. Different efficiency between $Mg^{2+}$ and polyamines revealed by our studies may be due to the fact that the cationic groups on the polyamines are spaced [47].

In vivo implication of present data seems to be interesting. For many decades the chloroplasts were isolated and restored to their maximal photosynthetic activity (100 μmol mg $Chl^{-1}$ $h^{-1}$) largely by the use of arbitrary combination of cations [23]. In the past, the major criterion for in vitro studies was more the simulation of the in vivo efficiency and less the simulation of the in vivo conditions, thus the concentration of $Mg^{2+}$ was saturating. Given that isolation procedures deplete small solutes that normally occur in the chloroplast, one should add them exogenously in order to restore their levels and study their effects; although one should pay attention also to the fact that isolation leads to the loss of stroma and the acceptor side of PSI. This contribution clearly shows that the higher polyamines (Spm

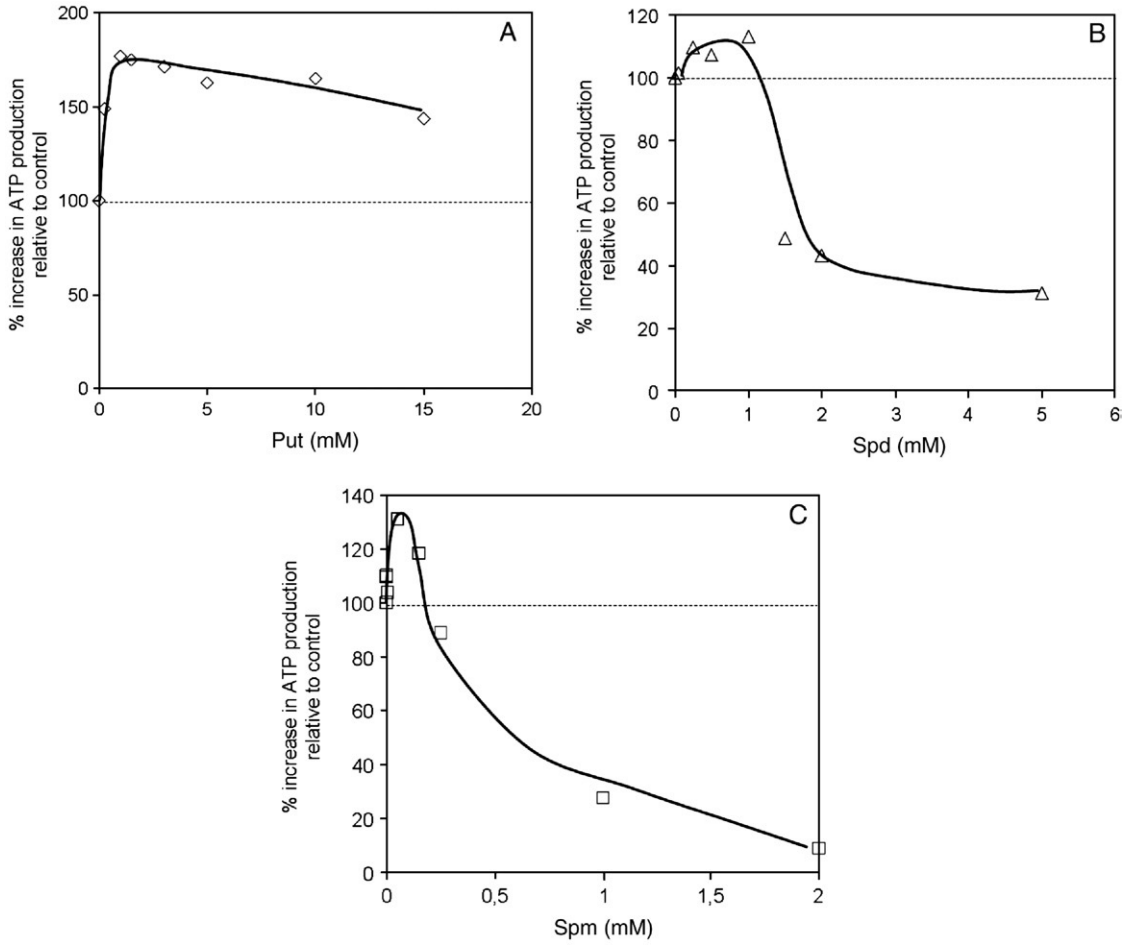


Fig. 9. Stimulation of ATP synthesis by different concentrations of Put (A), Spd (B) and Spm (C) in swiss-chard chloroplasts suspended in optimal-cation content media. Photophosphorylation was measured in a medium containing 200 mM sorbitol, 12 mM HEPES, 10 mM tricine, 10 mM KCl, 5 mM $MgCl_2$, 2 mM $K_2HPO_4$, 1 mM sodium ascorbate and 0.6 mM ADP (pH 7.8/KOH). 300 nM valinomycin was present while phenazine methosulphate (40 μM) was the electron acceptor (working concentration 28 μg Chl $ml^{-1}$). Values below dashed line in each panel represent uncoupling of photophosphorylation.

and Spd) have strong cationic effects. Polyamines and inorganic cations normally co-exist in chloroplast; we, thus, regard this study as a step towards a more precise evaluation of their roles. Perhaps this could explain the massive increase of polyamines during $K^+$ or $Mg^{2+}$ deprivation [5,48]. In general, Put accumulates in plants subjected to a wide range of stress conditions and it is not easy to provide a unifying hypothesis to explain the accumulation of Put in stress [5]. Based on our results, we suggest that Put is an efficient stimulator of ATP synthesis, which helps the organism to cope with stress through the increase of available ATP. This is in line with previous works reporting that putrescine significantly increases light energy utilization [9,11]. In addition, Spd and Spm can increase NPQ and activate photoprotection under relative innocuous light intensities with a mechanism that seeks elucidation. Last but not least, ATP synthesis could be rapidly regulated up or down by means of polyamine titer change. Namely Put elevation can boost energy synthesis, while Spm elevation can uncouple electron transport from ATP synthesis and act as a release valve for the thylakoid. All the above-mentioned traits support the view of an advanced role for plastidal polyamines in the bioenergetics of photosynthesis.

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## References

[1] S.S. Cohen, A Guide to the Polyamines, in: S.S. Cohen (Ed.), Oxford Univ. Press, New York, USA, 1998, pp. 1–624.
[2] C.W. Tabor, H. Tabor, Polyamines, Annu. Rev. Biochem. 53 (1984) 749–790.
[3] A. Bouchereau, A. Aziz, F. Larher, Martin-Tanguy, Polyamines and environmental challenges: recent development, Plant Sci. 140 (1999) 103–125.
[4] M.D. Groppa, M.P. Benavides, Polyamines and abiotic stress: recent advances, Amino Acids (Mar 14) (2007), doi:10.1007/s00726-007-0501-8.
[5] T.A. Smith, Polyamines, Annu. Rev. Plant Physiol. 36 (1985) 117–143.
[6] D. Dörnemann, E. Navakoudis, K. Kotzabasis, Changes in the polyamine content of plastidal membranes in light- and dark-grown wildtype and pigment mutants of the unicellular green alga *Scenedesmus obliquus* and their possible role in chloroplast photodevelopment, J. Photochem. Photobiol. 36 (1996) 293–299.
[7] G.F. Kramer, D.T. Krizek, R.M. Mirecki, Influence of photosynthetically active radiation and spectral quality on UV-B induced polyamine accumulation in soybean, Phytochemistry 31 (1992) 1119–1125.

[8] K. Kotzabasis, H. Senger, Free, conjugated and bound polyamines during the cell cycle in synchronized cultures of *Scenedesmus obliquus*, Z. Naturforsh. 49c (1994) 181–185.
[9] K. Kotzabasis, B. Strasser, E. Navakoudis, H. Senger, D. Dörnemann, The regulatory role of polyamines in structure and functioning of the photosynthetic apparatus during photoadaptation, J. Photochem. Photobiol. 50 (1999) 45–52.
[10] E. Navakoudis, C. Luetz, C. Langebartels, U. Luetz-Meindl, K. Kotzabasis, Ozone impact on the photosynthetic apparatus and the protective role of polyamines, Biochim. Biophys. Acta 1621 (2003) 160–166.
[11] L. Sfichi, N. Ioannidis, K. Kotzabasis, Thylakoid-associated polyamines adjust the UV-B sensitivity of the photosynthetic apparatus by means of light-harvesting complex II changes, Photochem. Photobiol. 80 (2004) 499–506.
[12] M. Sfakianaki, L. Sfichi, K. Kotzabasis, The involvement of LHCII-associated polyamines in the response of the photosynthetic apparatus to low temperature, J. Photochem. Photobiol., B Biol. 84 (2006) 181–188.
[13] G. Demetriou, C. Neonaki, E. Navakoudis, K. Kotzabasis, Salt stress impact on the molecular structure and function of the photosynthetic apparatus—The protective role of polyamines, Biochim. Biophys. Acta (BBA) — Bioenerg. 1767 (2007) 272–280.
[14] A. Bograh, Y. Gingras, H.A. Tajmir-Riahi, R. Carpentier, The effects of spermine and spermidine on the structure of photosystem II proteins in relation to inhibition of electron transport, FEBS Lett. 402 (1997) 41–44.
[15] R. Beauchemin, A. Gauthier, J. Harnois, S. Boisvert, S. Govindachary, R. Carpentier, Spermine and spermidine inhibition of photosystem II: disassembly of the oxygen evolving complex and consequent perturbation in electron donation from TyrZ to $P680^+$ and the quinone acceptors $Q_A^-$ to QB, Biochim. Biophys. Acta (BBA) — Bioenerg. 1767 (2007) 905–912.
[16] R. Beauchemin, J. Harnois, R. Rouillon, H.A. Tajmir-Riahi, R. Carpentier, Interaction of polyamines with proteins of photosystem II: cation binding and photosynthetic oxygen evolution, J. Mol. Struct. 833 (2007) 169–174.
[17] N.E. Ioannidis, L. Sfichi, K. Kotzabasis, Putrescine stimulates chemiosmotic ATP synthesis, Biochim. Biophys. Acta (BBA) — Bioenerg. 1757 (2006) 821–828.
[18] M. Holden, in: T.W. Goodwin (Ed.), Chlorophylls, Chemistry and Biochemistry of Plant Pigments, Academic Press, London, 1965, pp. 461–488.
[19] W. Bilger, O. Bjoerkman, Relationships among violaxanthin deepoxidation, thylakoid membrane conformation, and nonphotochemical chlorophyll fluorescence quenching in leaves of cotton (*Gossypium hirsutum* L.), Planta 193 (1994) 238–246.
[20] K. Maxwell, G. Johnson, Chlorophyll fluorescence. A practical guide, J. Exp. Bot. 51 (2000) 659–668.
[21] P. Horton, A. Hague, Studies on the induction of chlorophyll fluorescence in isolated barley protoplasts: IV. Resolution of non-photochemical quenching, Biochim. Biophys. Acta, Bioenerg. 932 (1988) 107–115.
[22] A. Melis, Spectroscopic methods in photosynthesis: photosystem stoichiometry and chlorophyll antenna size, Phil. Trans. R. Soc. Lond. 323 (1989) 397–409.
[23] D. Walker, Aqueous Phase Measurements, The use of oxygen electrode and fluorescence probes in simple measurements in photosynthesis, in: D. Walker (Ed.), Oxygraphics, Sheffield, UK, 1988, pp. 99–154.
[24] M. Avron, Photophosphorylation by swiss-chard chloroplasts, Biochim. Biophys. Acta 40 (1960) 257–272.
[25] E. Navakoudis, K. Vrentzou, K. Kotzabasis, A polyamine- and LHCII protease activity-based mechanism regulates the plasticity and adaptation status of the photosynthetic apparatus, Biochim. Biophys. Acta (BBA) -Bioenerg. 1767 (2007) 261–271.
[26] J. Barber, An explanation for the relationship between salt-induced thylakoid stacking and the chlorophyll fluorescence changes associated with changes in spillover of energy from photosystem II to photosystem I, FEBS Lett. 118 (1980) 1–10.
[27] M. Bernier, R. Carpentier, The action of mercury on the binding of extrinsic polypeptides associated with the water oxidizing complex of photosystem II, FEBS Lett. 360 (1995) 251–254.
[28] M. Bernier, R. Popovic, R. Carpentier, Mercury inhibition at the donor side of photosystem II is reversed by chloride, FEBS Lett. 321 (1993) 19–23.
[29] N.E. Holt, G.R. Fleming, K.K. Niyogi, Toward an understanding of the mechanism of nonphotochemical quenching in green plants, Biochemistry 43 (2004) 8281–8289.
[30] A.A. Pascal, Z. Liu, K. Broess, B. van Oort, H. van Amerongen, C. Wang, P. Horton, B. Robert, W. Chang, A. Ruban, Molecular basis of photoprotection and control of photosynthetic light-harvesting, Nature 436 (2005) 134–137.
[31] P. Horton, Light as an energy source and an information carrier in plant biology, in: R. Jennings, G. Zucchelli, F. Gheti, G. Combetti (Eds.), Non photochemical quenching of chlorophyll fluorescence, Plenoum Press, New York, 1996, pp. 99–112.
[32] D.M. Kramer, J.A. Cruz, A. Kanazawa, Balancing the central roles of the thylakoid proton gradient, Trends Plant Sci. 8 (2003) 27–32.
[33] H. Laasch, E. Weis, Photosynthetic control, 'energy-dependent' quenching of chlorophyll fluorescence and photophosphorylation under influence of tertiary amines, Photosynth. Res. 22 (1989) 137–146.
[34] H. Laasch, E. Weis, Differential sensitivity to dibucaine of photosynthetic control of electron transport and photophosphorylation in chloroplasts, Biochim. Biophys. Acta 936 (1988) 99–107.
[35] C. Giersch, Stimulation of photophosphorylation by low concentrations of uncoupling amines, Biochem. Biophys. Res. Commun. 100 (1981) 666–674.
[36] U. Pick, M. Weiss, The mechanism of stimulation of photophosphorylation by amines and by nigericin, Biochim. Biophys. Acta 934 (1988) 22–31.
[37] C. Sigalat, Y. de Kouchkovsky, F. Haraux, F. de Kouchkovsky, Shift from localized to delocalized protonic energy coupling in thylakoids by permeant amines, Biochimic. Biophys. Acta (BBA) — Bioenerg. 934 (1988) 375–388.
[38] T.D. Horner, E.N. Moudrianakis, The effect of permeant buffers on initial ATP synthesis by chloroplasts using rapid mixquench techniques, J. Biol. Chem. 258 (1983) 11643–11647.
[39] P.S. Brookes, Free Radical Biology and Medicine Mitochondrial $H^+$ leak and ROS generation: An odd couple 38 (2005) 12–23.
[40] J. Barber, Influence of surface charges on thylakoid stucture and function, Annu. Rev. Plant Physiol. 33 (1982) 261–295.
[41] H.W. Trissl, C. Wilhelm, Why do thylakoid membranes from higher plants form grana stacks? Trends Plant Sci. 18 (1993) 415–419.
[42] H. Kirchhoff, H. Winfried, S. Haferkamp, T. Schoot, M. Borinski, U. Kubitscheck, M. Rögner, Structural and functional self-organization of Photosystem II in grana thylakoids, Biochim. Biophys. Acta 1767 (2007) 1180–1188.
[43] J. Standfuss, A.C. Terwisschavan, M. Lamborghini, W. Kuehlbrandt, Mechanisms of photoprotection and nonphotochemical quenching in pea light-harvesting complex at 2.5Å resolution, EMBO J. 24 (2005) 919–928.
[44] S. Bose, C.J. Arntzen, Reversible inactivation of Photosystem II reaction centers in cation-depleted chloroplast membranes, Arch. Biochem. Biophys. 185 (1978) 567–575.
[45] J.-M. Briantais, C. Vernotte, J. Olive, F.-A. Wollman, Kinetics of cation-induced changes of photosystem II fluorescence and of lateral distribution of the two photosystems in the thylakoid membranes of pea chloroplasts, Biochim. Biophys. Acta 766 (1984) 1–8.
[46] J.A. Nairn, W. Haehnel, P. Reisberg, K. Sauer, Picosecond fluorescence kinetics in spinach chloroplasts at room temperature, effects of $Mg^{2+}$, Biochim. Biophys. Acta 682 (1982) 420–429.
[47] J.C. Allen, Biochemistry of the polyamines, Cell Biochem. Funct. 1 (1983) 131–140.
[48] N.D. Young, A. Galston, Physiological control of arginine decarboxylase activity in $K^+$ deficient oat shoots, Plant Physiol. 76 (1984) 331–335.